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# Biochemistry of Ischemic Reperfusion Injury

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Dermal microvascular endothelial cells when exposed to hypoxic conditions show a rapid induction of several proteins that do not increase in other cell types exposed to the same conditions of hypoxia. These new proteins differ from stress proteins, are induced rapidly, and are expressed transiently. They include a group of acidic proteins with molecular weights in the range of 100-120,000 and a least one glycoprotein. This response is accompanied by a transient overall increase in protein synthesis as determined by the incorporation of radio labeled methionine. The changes seen in proteins synthesized by dermal microvascular endothelial cells takes place in the same time scale as ischemia-reperfusion injury and may reflect the specialized change of functions of the microvasculature observed under conditions of hypoxic stress in vivo.

Since inflammation following ischemia is responsible for the subsequent tissue pathology, the sequential changes in the expression of four protooncogenes, c-*fos*, c-*myc*, c-*sis* and H-*ras* were determined following activation with the inflammatory mediator phorbol myristate acetate. Differences in the extent of activation and kinetics of each oncogene were determined. Phorbol myristate acetate activates protein kinase C, and suggests a close link between activation of protein phosphorylation, regulation in the synthesis of c-*myc*, c-*sis*, and c-*fos* mRNA and changes in the morphology and inflammatory response of dermal microvascular endothelial cells.

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## A. INDUCTION OF OXYGEN-REGULATED PROTEINS IN MICROVASCULAR ENDOTHELIAL CELLS

### BACKGROUND TO THE STUDIES

In a wide variety of rodent and human cells, hypoxia causes induction of a well defined group of proteins. These proteins, termed oxygen-regulated proteins (ORPs), have molecular weights of approximately 260,000, 150,000, 100,000, 80,000, 60,000, 57,000, 47,000, 34,000 and 33,000. Some of these proteins have been related to other stress responses. Thus, glucose deprivation and the treatment with a variety of toxic drugs causes induction of a series of proteins termed glucose-regulated proteins (GRP) of which two have been shown to be identical to ORPs (ORP80 with GRP78, ORP100 with GRP94). In addition, GRP78, and by inference ORP80, has been identified as immunoglobulin heavy chain binding protein and ORP34 as LDH K. ORP33, that is induced by a wide variety of agents, and environmental conditions, including oxidative stress, heavy metals metabolic inhibitors, has been shown to be hemoxygenase.

The rapidity of the induction of ORPs during hypoxia varies. ORP260 may be induced after as little as 1 h, but the increased synthesis of the others only becomes evident after 2-4 h. In general, the maximal rate of synthesis is achieved by 12 h. Under inducing conditions other than hypoxia, the time course of their appearance may be quite different.

ORPs are distinguishable from the stress proteins induced by thermal shock (heat shock proteins, HSP) which generally are repressed by hypoxia. However, hypoxic induction of certain HSP in coronary and hippocampal endothelial cells has been reported. Brief induction of HSP following establishment of hypoxia has been observed in several tumor cells line.

The induction of proteins by hypoxia which differ from the known ORPs, has been reported in bovine large blood vessel endothelial cells. In general, expression of these proteins increased progressively over 24-48 h.

Endothelial cells lining large blood vessels are known to differ from those of the microvasculature and from one tissue to another. There are also notable species differences. In these studies, we have concentrated on the induction of ORP in human dermal microvascular endothelial cells, which are important in hypoxic responses following injury. In these cells we observed the rapid induction of novel intracellular and surface proteins which are, not found in other endothelial cell types and on a time scale comparable with the physiological responses to hypoxia.

### METHODS USED TO STUDY PROTEIN INDUCTION

*1. Cells and Culture*

Human dermal microvascular endothelial cells were isolated from newborn foreskins by thin layer perfusion as described previously. The cells were cultured in Iscove's medium, supplemented with $5x10^{-4}$ M dibutyryl cAMP and $3x310^{-5}$ M isobutyl methylxanthine. 8% fetal bovine serum and 2% human prepartum serum. All cultures were used at passage 4 or 5. Umbilical vein endothelial cell were purchased from Clonetics Corp. and cultured according to the manufacturer's instruction.

*2. Means of hypoxia*

Cultures were placed in specially designed chambers attached to a gas and vacuum manifold and repeatedly flushed with 95% nitrogen, 5% $CO_2$ by evacuation. The chambers were maintained at 37° in a water bath. In the first flushing cycle, the pressure was reduced to 386 mm Hg, reducing the oxygen tension to 10% atmospheric, and then restored to 600 mm Hg with nitrogen/$CO_2$. After 7 min., the pressure was then reduced to 185 mm Hg and brought back to 600 mm Hg. Two such cycles reduced the oxygen tension by a factor of 10. The standard protocol included 9 cycles, at which point the partial pressure of oxygen was reduced to 0.04% of atmospheric as measured by a polarographic oxygen electrode (Controls Katharobic, Gulph Mills, PA). The process of establishing hypoxia took 63 min., although 3% oxygen (7.6 mm Hg), which gave about 50% of the maximal inhibition of protein synthesis after 24 h, was reached after 14 min. The cultures were incubated under these condition for varying times from 2 h to 24 h, measured from the end of the final cycle of evacuation and flushing, and opened to the atmosphere before metabolic labeling. The pH of the medium was unaffected by this procedure. Control cultures were maintained in air/5% $CO_2$ at 37°.

*3. Cell labeling and fractionation*

Cultures were labeled with 50 μCi/mL [$^{35}$S]-methionine in methionine-free DMEM supplemented with glutamine and dialyzed fetal bovine serum for 1 h at 37°C under normoxic conditions. After removing the medium, cells were harvested in 100 μL lysis buffer (10 mM Tris, 150 mM NaC1, 10 μg/mL DNase, 0.5μg/mL leupeptin, 0.7 μg/mL pepstatin, 1 mM PMSF, 2.1 μg/mL aprotinin, 1% triton X-100, pH 7.6) and centrifuged at 15,000 rpm in a microfuge at 4°C for 6 min. A volume of the supernatant containing an amount of protein constant throughout a single experiment was brought to 9 M urea in IEF sample buffer and separated by 2-dimensional electrophoresis. The IEF gel contained 80% pH-4-7 ampholenes and 20% pH 3-10 ampholenes and the pH range of the gel was measured to be 4.6-7. The second dimension was run in 8% acrylamide under discontinuous buffer conditions. Gels were either soaked for 30 min. in 16% sodium salicylate, dried and fluoragraphed, or electroblotted to PVDF membranes and stained with UEA-1. Electroblots were blocked with 1% Tween-20 in PBS overnight at room temperature and then incubated with 10 μg/mL biotinylated UEA-1 in 1% Tween-20 in PBS for 1 h at room temperature with rocking. The blot was then washed 3 times with 1% Tween-20 in PBS. After incubation with streptavidin-phosphatase at room temperature with rocking for 30 min., the blot was washed 3 times as before and incubated with 0.5 mM 5-bromo-4-chloroindoly phosphate, 0.3 mM nitroblue tetrazolium in 0.1 M NaC1, 50 mM $MgC1_2$, 0.1 mM tris, pH 9.5 until the pattern developed. All blots were developed for the same length of time. Radioautographs were quantitated using an Applied Biovision Lynx.

*4. Lactate dehydrogenase assay*

Lactate dehydrogenase was estimated by measuring oxidation of 0.8 mM $NADH_2$ by 4 mM sodium pyruvate in 0.1 M sodium phosphate buffer, pH 7.5 at 340 nm.

MAJOR FINDINGS OF THESE STUDIES

*1. Individual Variation of 2-dimensional patterns of proteins from DMEC*

Since the data reported here were all obtained by the use of primary cultures, we have compared cultures from 12 individuals to evaluate the extent of individual variation. In general, the patterns of labeling was similar in all individuals.

*2. Variation of 2-dimensional protein patterns with endothelial cell origin and morphology*

Endothelial cells from varying anatomical sites have been reported to show differences in surface markers, cytokine responses, adhesion molecules and growth factor receptors. We compared the 2-dimensional gel electrophoretic patterns of proteins extracted from DMEC, spindle-shaped cells derived from DMEC by omission of cAMP from the medium, and UVEC. Of 181 proteins analyzed quantitatively on these gels, 31 increased by a factor greater than three in epithelioid cells and 21 decreased by a similar amount. In particular, a major protein at pI 6.4 and molecular weight 55,000 which sometimes appears as a horizontal streak, is present in the epithelioid cultures but reduced or absent from the spindle-shaped cells. This result has been obtained in 10 experiments. The protein is interesting s it has been shown to possess UEA-reactive glycosylation and is probably a microvascular endothelial cell surface protein.

The UVEC cultures were obtained from different individuals, but consistent differences in two-dimensional gel patterns from DMEC have been detected in 3 individuals. The epithelioid DMEC-specific protein described above in absent in UVEC but is replaced by a characteristic set of 3 new proteins.

*3. Viability of DMEC under hypoxia*

No morphological change was seen in DMEC up to 24 h of hypoxia and the number of cells remained constant. Cell lysis was assessed by measurement of lactate dehydrogenase activity in the supernatant and the cells. No evidence was found for cell lysis up to 24 h of hypoxia.

*4. Dependence of protein synthesis on hypoxia*

Overall incorporation [$^{35}$S]-methionine into detergent-extractable protein under conditions of severe hypoxia (0.04% oxygen) showed an initial increase of about 80% in 3 of 5 examples, followed in all cases by a decline to 35% of control. The early stimulation occurred within the first 2 h and incorporation remained elevated for at least 4 h. This behavior differs from other cell types. however, by 8 h , amino acid incorporation decreased and remained low for at least 24 h. The initial increase in amino acid incorporation seen in some epithelioid DMEC preparation has never been detected in UVEC nor in DMEC-derived spindle-shaped cells.

The inhibition of methionine incorporation seen after 24 h at 0.04% oxygen also occurs at lesser degrees of hypoxia. The oxygen tension giving half maximal response was approximately 3% atmospheric pressure.

*5. Induction of proteins in DMEC by hypoxia*

The proteins synthesized in reoxygenated cells after various times of hypoxia were analyzed by 2-dimensional gel electrophoresis. The gels were loaded with 16.6 $\mu$g $^{35}$S-methionine-labeled, triton-soluble protein from aerobic cells or cells incubated under hypoxia for various times.

Within 2 h of hypoxia, there was a striking induction of a series of acidic proteins with molecular weights in the range 100,000-12,000. Induction of these proteins has not been detected in any of ten lines of rodent and human cells so far analyzed nor in spindle-shaped cells nor UVEC. Detailed comparison of proteins labeled in cells exposed to hypoxia for 2 h with those from normoxic cells showed that, 84 proteins analyzed, 21 proteins were induced by hypoxia by a factor greater than three and three proteins were repressed. The proteins that were induced by hypoxia in non-DMEC cells, particularly ORP 80, changed in DMEC by less than a factor of three at times less than 24 h. We regard a factor of three as the confidence limits of this experiment.

It should be noted that the level of constitutive expression of the acidic 100k-12k proteins varies from one cell preparation to another and they are frequently present in UVEC.

The VORP detected on these 2-dimensional gels are not detected on UEA-stained blots and, therefore, appear to be non-glycosylated, intracellular, proteins.

*6. Surface protein expression DMEC under hypoxia*

Since early stages in inflammation following hypoxia in vivo involve interaction of leukocytes with endothelial cells, hypoxia-induced changes in DMEC surface proteins are of particular interest. These were studied by staining blots of proteins separated by 2-dimensional electrophoresis with *Ulex europeus* agglutinin (UEA-1). This lectin was selected because it binds to many DMEC glycoproteins specifically. Striking changes were seen in the protein marked HU-1. A protein occurred in the same position on [$^{35}$S]-methionine-labeled gel at pI 5.1, Mr 57,000. This protein showed hypoxia-induced increases in intensity prior to the changes in the UEA-defined spot.

SIGNIFICANCE OF THESE STUDIES

This study describes the response of human dermal microvascular endothelial cells to hypoxia. With respect to cellular swelling and cell death, we were unable to detect morphological changes or cell lysis during period of hypoxia up to 24 h. Many mammalian cells are able to survive prolonged hypoxia in this way and we regard it unlikely that cell death plays any major direct role in the tissue damage that follows hypoxic episodes.

The observation of an increase in protein synthesis at early times of hypoxia occurred in 3 of the 5 experiments reported here. It accompanied the induction of microvascular VORPs, but it is unlikely that an increase in this small number of proteins could account for the overall stimulation of methionine incorporation that presumably reflects a transient overall increase in protein synthesis. It is, however, variable and only occurs in a proportion of the primary cell strains studied here. This is in keeping with the overall variability that we have found in primary DMEC cultures derived from different individuals.

The major hypoxia-induced proteins in these experiments, particularly an acidic group with molecular weights in the range 100,000-120,000, pI about 5.1, are induced very rapidly after exposure of the cells to hypoxic conditions. The changes are easily detectable at 2 h, the earliest time used and reach maxima within 2-4 h. The precise timing is uncertain because of the time taken to reach the conditions of extreme hypoxia used in these experiments and the oxygen tension at which the response is initiated. Nonetheless, by 8-24 h the hypoxic DMEC responses decline almost to unstimulated

synthesis rates. The time course is also more rapid than that observed with most of the ORPs that generally reach peak synthesis rates by 12 h, although ORP260 can be detected within 2 h.

In experiments to investigate glycosylated proteins, presumably expressed on the cell surface, we detected a major protein, with pI 5.8 and molecular weight 70,000. This protein is prominent in the UEA stained gels, but much less evident on [$^{35}$S]-methionine-labeled gels where it is usually only detected by its distortion of surrounding spots. This implies that its concentration is very high, although it appears to contain little methionine, and it may well account for much of the characteristic UEA-I reactivity of the human microvascular endothelium. It may be noted that the pI~5, 100,000-115,000 Mr ORPs detected on [$^{35}$S]-methionine-labeled gels are not represented on the UEA-stained gels. They are, therefore, not fucosylated and probably do not occur on the cell surface. However, actin, to our surprise, was consistently stained to a small extent on our blots implying some degree of glycosylation. The lectin binding appeared to be specific in that other highly expressed proteins showed no reaction with UEA.

Hypoxia induced changes in HU-1, as detected by UEA staining, show an interesting relationship with changes seen in the [$^{35}$S]-methionine-labeled gel. The UEA-stained blot detects the total amount of protein present, while the signal on the [$^{35}$S]-methionine-labeled gel is related to the amount of incorporation during 1 h incubation with the radioactive precursor, and represents the rate of synthesis of the protein. At 2 h, the amount of HU-1 detected by UEA reached a maximum, with a corresponding increase in the intensity of the [$^{35}$S]-methionine-labeled spot. At 4 h, the amount detected by UEA had decreased, and the corresponding spot on the [$^{35}$S]-methionine-labeled gel changed character, from a triple to doublet. At later times, the [$^{35}$S]-methionine-labeled spot resumed its triplet appearance, returning to close to normal at 24 h. The rate of synthesis therefore appeared to undergo a rapid increase followed by a rapid decline to approximately its original rate. The qualitative changes seen in the [$^{35}$S]-methionine-labeled gel may reflect post-translational modifications, including the secondary glycosylation that is the target of UEA-I. HU-1 is very similar in electrophoretic to vimentin, a major endothelial cell cytoskeletal protein of the intermediate filament system, that is known to undergo modifications during transdifferentiation of endothelial cells in response to cytokines or culture conditions.

Several pieces of evidence have related the hypoxic response of smooth muscle cells to a decline in cellular cAMP, and a similar phenomenon has been implicated in the increased permeability of bovine endothelial cell cultures. The DMEC used in the experiments reported here were cultured in $5x10^{-4}$M dibutyryl cAMP and $3x310^{-5}$ M isobutyl methylxanthine to maintain optimal growth in the epithelioid conformation and Factor VIII expression. However, we have obtained identical results in terms of hypoxia-induced protein expression in cells grown in cAMP but exposed to hypoxia in the absence of cAMP. In our experience with this cell type, cAMP appear not to be involved in the induction of the proteins reported here.

The induction of the DMEC proteins by hypoxia, detected in these experiments, appears to identify a unique response of these cells to hypoxic stress. The response differs from other cell types both in the proteins induced and in the minor responses of the usual ORPs. The relationship of the VORP to the role of microvascular cells in hypoxia-induced tissue injury is of great interest. The most striking proteins seen on [$^{35}$S]-methionine-labeled gels are apparently non-glycosylated and therefore probably

intracellular. It is unlikely that they play a direct role in the diapedesis of neutrophils. HU-1, however, is glycosylated and may lie on the surface of the cells and play a role in cellular adhesion and other intercellular interactions.

These results provide an accessible model for the hypoxic responses of microvascular endothelial cells in other locations such as heart and brain where ischemia can lead to grave results. It will be important to determine whether the proteins described here can be detected in capillary endothelial cells isolated from other tissues sensitive to hypoxia and to identify their roles in the endothelial cell response that occurs in ischemic organs.

## B. PROTOONCOGENE INDUCTION IN MICROVASCULAR ENDOTHELIAL CELLS.

### BACKGROUND TO THE STUDIES

Endothelial cells lining blood vessels from part of the reticuloendothelial system. They act both as a non-thrombogenic surface and also play a crucial role during inflammation. In the presence of a variety of cytokines, cellular adhesion molecules such as ICAM-1 and ELAM are induced. These molecules allow binding of lymphocytes, macrophages, and polymorphonuclear leukocytes in the earliest stages of the inflammatory process as occurs during hypoxia. In other circumstances, for example, following injury or in the presence of a tumor, endothelial cells will cooperate in angiogenesis. This process includes migration, mitosis, the formation of new blood vessels, and anastamosis.

Endothelial cells from different sources have been found to vary in their properties in relation to the physiological function of the tissue of origin. Endothelial cells of the dermal microvasculature are of particular importance following ischemia. For these reasons, all the experiments in this study have been carried out with dermal microvasculature endothelial cells.

Protein kinase C (PKC) is an enzyme of high activity by comparison with other cellular protein kinases. PKC has been implicated in the control of cell proliferation and differentiation as a late component of the cellular signal processing system. In a number of cases it is thought to cause changes in the properties of major cellular components directly. For instance, the ability of PKC to phosphorylate laminin and vimentin may be related to dissociation of the nuclear membrane and the intermediate filament cytoskeleton during reorganization and mitosis. Protein kinase C also inactivates the EGF (epidermal growth factor) receptor tyrosine kinase by phosphorylation, apparently as part of a feedback control mechanism. Activation of protein kinase C in endothelial cells causes a characteristic transformation to a spindle shape. The possible significance of the morphological changes, in terms of angiogenesis, inflammatory function, and ischemic injury is unknown. In these studies, we have concentrated on four cellular proto-oncogene markers associated with growth and protein kinase C activation.

### METHODS USED IN THESE STUDIES

Human microvascular endothelial cells were isolated from neonatal foreskins. To obtain sufficient cells for consistent cultures, frozen stocks from six individuals were pooled. Experiments were carried out with cells at passage 5. TREND cells (SV-40 transformed human microvascular endothelial cells) and cultures which had spontaneously converted to a spindle shaped morphology were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Subconfluent 100 m m dishes were treated with 2 $\mu$M PMA and harvested at different time points. All cultures were harvested within 30 minutes.

The dishes were drained and the cells dissolved in 4M guanidine thiocyanate, 0.1 M 2-mercaptoethanol. Cell extracts were centrifuged through a 5.7 M CsCl cushion at 36,000 rpm in a Beckman SW60 rotor for 24 hours at 20°C. Supernatant solutions were removed and the pellets washed with 70% ethanol, then dried in vacuo. The dried pellets were dissolved in water, and the RNA precipitated with 2.5 volumes of ethanol in 0.2 M potassium acetate, pH 5.1.

RNA was collected, quantified, and 15 μg portions electrophoresed on 1.2-1.5% agarose gels containing 2.2M formaldehyde. Gels were processed as follows: one 20 minute wash with 60 mM NaOH, two rinses with 10 X SSC, and capillary transfer to Optibind NC (Schleicher and Schuell, Keene, NH) in 10 X SSC. The RNA was cross-linked to the membrane with ultraviolet irradiation using a Stratalinker (Stratagene, La Jolla, CA). Membranes were pre-incubated at 67°C in 2.5 X SSC, 0.1% sodium pyrophosphate, 0.1% sodium dodecyl sulfate, 5 X Denhardt's solution for 2 hours, with a further 3 hour incubation after the addition of 10 μg/ml herring sperm DNA and 10 μg/ml poly-(A). The membranes were then incubated overnight at 67° with individual radioactive proto-oncogene probes in the prehybridization solution, supplemented with 10% dextran sulphate. Radioactivity was incorporated into the probes using either random priming or nick translation. Filters were washed under stringent conditions (0.1X SSC at 67°C) and autoradiographed on Kodak X-OMAT AR film (Kodak, Rochester, NY). Radioautographs were quantitated with a Bio-Rad model 620 Video Densitometer or a Bio-Vision BVI4000, using two-dimensional methods. Densitometry values were normalized to β-actin. In this series of experiments, the cellular β-actin mRNA content, measured on lanes loaded with the same amount of total RNA, was found to decline at a rate of 0.84% +/-1.2% over the time of experiment, which was not significant at $p<0.4$ and has been ignored.

MAJOR FINDINGS OF THESE STUDIES

1. Phorbol Ester Induced Morphological changes

Treatment of endothelial cell cultures for 16 hours with 2 μM phorbol myristate acetate (PMA) caused tesselated, polygonal cells to become refractile, withdraw from their neighbors and begin a transition to spindle shaped morphology. After a longer period of culture, they became fibroblast-like.

2. Induction of *C-fos* by Phorbol Ester

Transcription of *c-fos* was absent in untreated microvascular endothelial cells, but was rapidly induced following the addition of 2 μm PMA. Transcripts first appeared at 30 minutes and reached a peak by 1 hour. By 2 hours, *c-fos* mRNA was no longer expressed and showed no subsequent induction within 32 hours.

3. *C-myc* Repression by Phorbol Ester

Following treatment with 2 μM PMA, *c-myc* expression declined with an apparent half-like of 4.4 hours +/-19% over the first 8 hours. In contrast, TREND cells showed a 5.7-fold transient stimulation, which reached a maximum at about 2 hours. Initial experiments with TREND cells were carried out in the medium, DMEM, in which they were grown. Since this differs markedly from that used for endothelial cells, notably in the absence of dibutyryl cAMP and isobutylmethylxanthine, the experiments were repeated in modified Iscove's medium, containing these components. Under these conditions, the induction, although reduced 80%, was still observed.

4. Inhibition of *c-sis* Expression by Phorbol Ester

Expression of *c-sis* was prominent in cultured endothelial cells. Following treatment with 2$\mu$ PMA, *c-sis* expression declined 68% +/-6% in 16 hours. Similarly, when cAMP was withdrawn from the growth medium *c-sis* expression fell by 64%.

5. Changes in Other Proto-oncogenes

*H-ras* was found to be present in cultured endothelial cells but showed no change after treatment with 2 $\mu$M PMA. *N-ras* was not detected under any experimental condition.

SIGNIFICANCE OF THESE STUDIES

Treatment of endothelial cells with phorbol ester causes a series of changes in morphology and gene expression. Protein kinase C is an element of the cellular signal transduction system which lies functionally in an intermediate position between cell surface receptors and gene expression. The isoenzyme species of PKC are activated by diacyl glycerol to different extents. Diacyl glycerol is produced by phospholipase C, which is activated by receptor-mediated events in the cell membrane and by calcium. Calcium may be released from intracellular stores, a process in which inositol triphosphate, also a product of phospholipase C, is particularly important. Calcium may also be brought in through the cell membrane by activation of calcium ion channels. In the overall signal transduction system, protein kinase C lies downstream of the activity of the *ras* gene product and upstream of calmodulin, which is phosphorylated by PKC. PKC is a component of feedback control loops in some cells. For instance, PKC modifies by phosphorylation the activity of the EGF receptor. Protein kinase C is known to phosphorylate vimentin, apparently causing dissolution of the intermediate filament cytoskeleton. Fragments of the PKC enzyme phosphorylate histone. Non-physiological stimulation of protein kinase C may cause an uncoordinated activation of parts of the cellular signal transduction system.

Protein kinase C activation is observed both in pathways which stimulate *c-sis* expression, as IL-1 and thrombin and in pathways which inhibit it, as with $\gamma$-interferon. The effect of these cytokines on *c-sis* expression depends also other control pathways, where protein kinase C does not participate. The difference between the bovine and human cells may lie in the relative activation of these other control pathways under the condition of culture.

The half-life for the loss of *c-sis* mRNA is about 12 hours, which compares with a value of 70-90 minutes reported in cAMP treated cells. Unless the mRNA is markedly stabilized under these conditions, the decline in expression appears to result from a gradual and progressive repression of transcription. This may be secondary to the change in differentiation reflected by the morphological observations.

The transient induction of *c-fos* following PMA addition is typical for many cell types but differs from macrophages in which expression is constitutive types but differs from macrophages in which expression is constitutive. In this respect, then, cultured microvascular endothelial cell show a property unlike macrophages. *C-fos* appears to play a role in many cellular processes. These include binding to AP-1 sites in the promoter regions of numerous genes, several of which code for differentiation products. The *c-fos* gene product plays roles both in proliferation and differentiation. It is a complete oncogene, that is, able to cause transformation and unlimited growth without accessory oncogenes. It is also found in several cell types undergoing terminal differentiation, such as suprabasal epidermal cells.

Under the conditions of our experiments, the amount of *c-myc* mRNA decline steadily following addition of PMA, despite the stimulation of *c-fos*. The response in DMEC, however, contrasts with results from the SV-40 transformed derivatives of human dermal microvascular endothelial cells, which show transient induction. In addition to the presence of SV-40, the TREND cell line differs from primary dermal endothelial cells in the lack of sensitivity of their morphological phenotype to cAMP. Although the induction of *c-myc* in TREND cells is apparently quantitatively sensitive to cAMP, the repression of *c-myc* in DMEC appears to involve a different cellular control system. In the case of DMEC, cAMP is required for the maintenance of the epithelioid morphology, but does not interfere with multiplication. DMEC also differ from renal microvascular endothelial cells in their response to cAMP. The basis for this differential response has yet to be defined.

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